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(54) Method and composition for the evaluation of phagocytic response

(57) A method and composition for measuring the ability of an organism to resist infection are described. A sample of blood cells is taken from an organism. The phagocytic activity of the phagocytes in the blood is estimated by mixing the cells with zymosan particles or polymeric beads coated with protein and a luminescent chemical. When the phagocytic cells are mixed with either of these compositions, the cells engulf the particles thus causing the activation of the cells' biochemical mechanisms. Oxygen intermediates result from this mechanism causing a reaction with the luminescent chemical resulting in the production of light. This light is measured on a luminometer and based on the ratio of light production and the maximum produced, the phagocytic activity of said cells is measured.

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Fig. 1 TYPICAL RESPONSE OF DILUTED WHOLE BLOOD CHEMILUMINESCENT ASSAY USING ZYMOSAN BASED REAGENT.

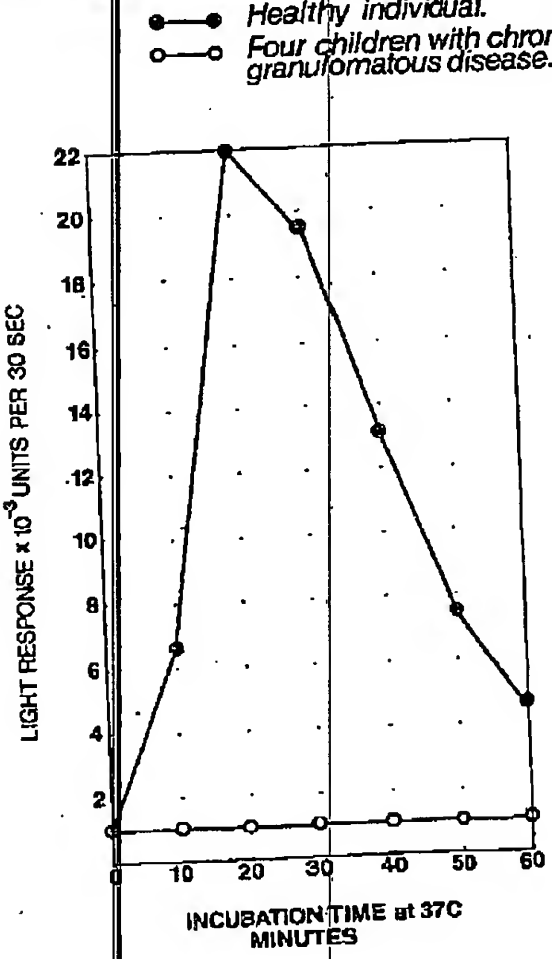

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Fig.2 TYPICAL RESPONSE OF ISOLATED NEUTROPHILS-ASSAY USING ZYMOSAN BASED REAGENT.

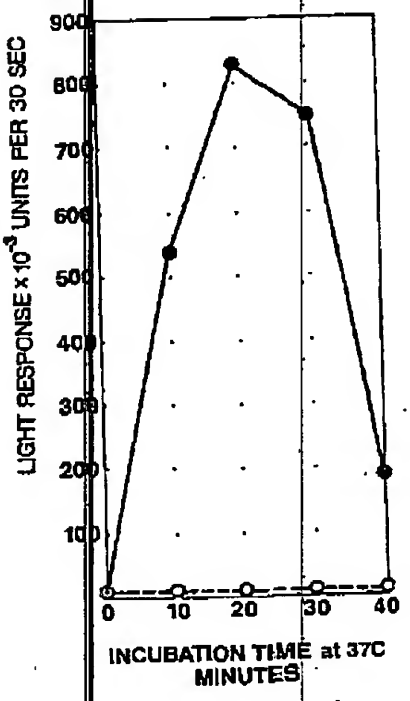

Fig.3 TYPICAL RESPONSE OF ISOLATED NEUTROPHILS-ASSAY USING POLYMERIC BEAD BASED REAGENT.

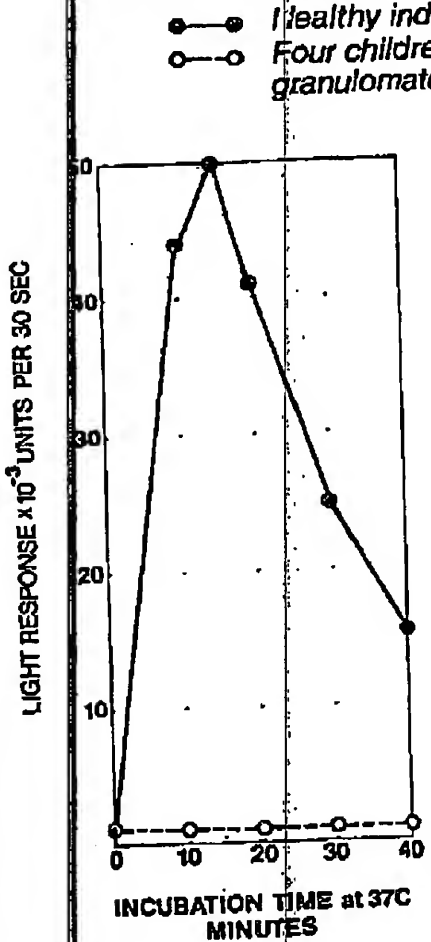

Fig.4 TYPICAL RESPONSE OF DILUTED WHOLE BLOOD CHEMILUMINESCENT ASSAY USING ZYMOSAN BASED REAGENT.

●—● *Average of one adult and two asthmatic children not on medication.*

o----o *Average of two children on therapeutic doses of theophylline for asthma.*

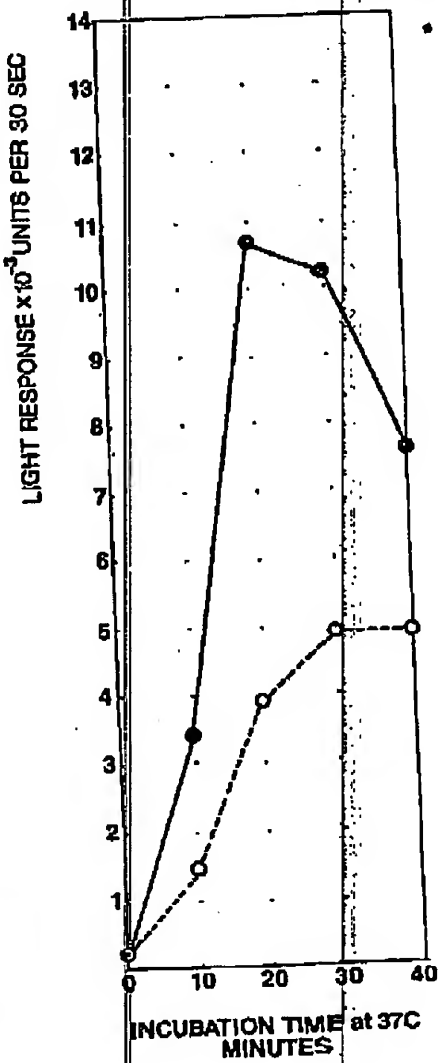

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Fig.5 TYPICAL RESPONSE OF ISOLATED NEUTROPHILS-ASSAY USING ZYMOSAN BASED REAGENT.

●—● *Average of asthmatic(no medication) children and adults.*

○—○ *Average of two asthmatic children on therapeutic doses of theophylline.*

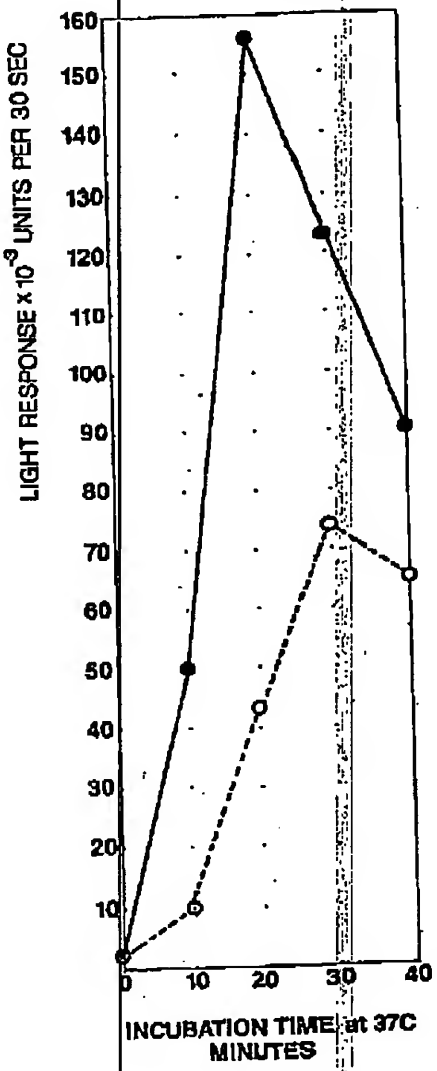

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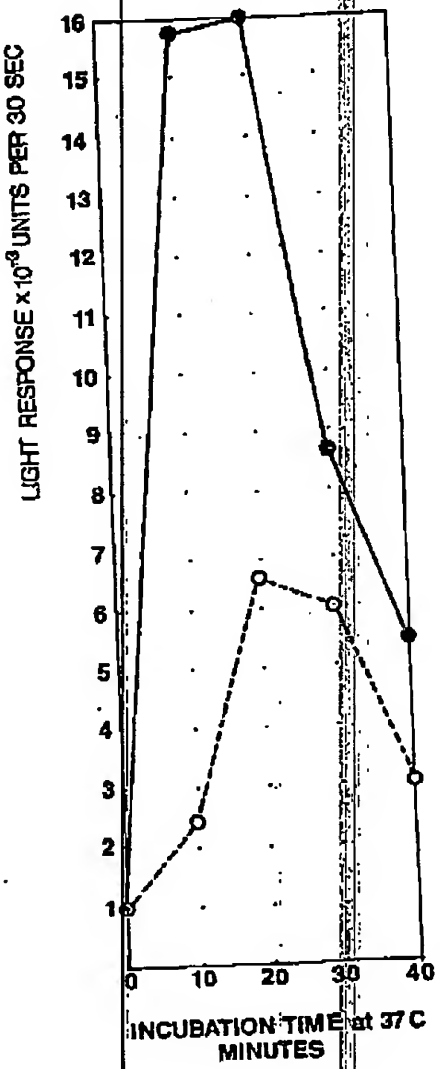
Fig.6 TYPICAL RESPONSE OF ISOLATED NEUTROPHILS-ASSAY USING POLYMERIC BEAD BASED REAGENT.

Average of asthmathic(no medication) children and adults.

Average of two asthmatic children on therapeutic doses of theophylline.



SPECIFICATION

Method and composition for the evaluation of phagocytic response

Technical Field

The field of art to which this invention pertains is assay methods of measuring the ability of an organism to resist infection, compositions useful therefor, and methods of making such compositions.

Background Art

The observation that certain phagocytic cells found in the blood and in organs such as the lung generate light as a result of the physical ingestion of particles such as bacteria or non-living compositions has been published over the years in the scientific literature. These observations are based on specific cells found in the blood or organs which are known to serve a critical function of seeking out, phagocytosing (engulfing) and killing such particles such as bacteria through complex chemical mechanisms. These cells may also seek out and remove non-infectious agents such as airborne particulate pollutants. Part of this cellular chemical mechanism includes the production of relatively high energy oxygen intermediates. The cell will produce these chemicals when stimulated to phagocytize. These high energy oxygen intermediates will decay to lower energy levels and in the process light will be generated. Although these low levels of light are detectable using instruments such as liquid scintillation spectrometers, this is a very cumbersome technique which does not permit rapid analysis of a large number of samples or samples with a large number of variables to be investigated. And, while this phenomenon has been known for some time, little advantage has been taken of it due to the limitations described above along with nonavailability of relatively stable reagents.

Disclosure of Invention

The present invention is directed to a method of measuring the phagocytic response of various phagocytic cells. Such method takes advantage of the process of phagocytosis used by an organism to resist infection or to remove particulate matter from the lungs, etc. A sample of blood-derived or organ-derived phagocytic cells is taken from a human or another animal and a particle-containing material added thereto which is engulfed by the phagocytic cells. The cells respond to the particle-containing material and generate oxygen intermediates which in turn, react with a chemical in the particle-containing material. This reaction generates light as a result. This light so produced is detected and measured.

Another aspect of the invention includes one such particle-containing reagent adapted to being phagocytized by the cells and comprises polymeric beads coated with proteins to which a luminescent chemical such as 5-amino-2, 3,dihydro-1, 4-phthalazinedione (luminol) is bonded thereto.

Another aspect of the invention includes another such particle-containing reagent adapted to being phagocytized by the cells and comprises polysaccharide particles derived from yeast cell walls (zymosan) adsorbed with proteins to which a luminescent chemical (luminol) is admixed.

Another aspect of the invention includes a method of making the coated, particulate, polymeric beads according to the present invention. The polymeric beads, about 2 to about 9 microns in diameter are coated with a protein and luminescent chemical. The luminescent chemical can be coated on the protein or admixed with the protein and the two applied to the polymeric beads together. The resultant particles are washed, resuspended to a final concentration and lyophilized.

Another aspect of the invention includes a method of making the coated zymosan particles useful according to the present invention. The zymosan particles are coated with a protein. Luminol is mixed with the coated particles and the resultant material is lyophilized into a stable, homogeneous product.

The foregoing, and other features and advantages of the present invention, will become more apparent from the following description and the accompanying drawings.

Brief Description of the Drawings

Figure 1 shows a typical response of diluted whole blood chemiluminescent assay using zymosan based reagent.

Figure 2 shows a typical response of isolated neutrophils-assay using zymosan based reagent.

Figure 3 shows a typical response of isolated neutrophils-assay using polymeric bead based reagent.

Figure 4 shows a typical response of diluted whole blood chemiluminescent assay using zymosan based reagent.

Figure 5 shows a typical response of isolated neutrophils-assay using zymosan based reagent.

Figure 6 shows a typical response of isolated neutrophils-assay using polymeric bead based reagent.

Best Mode for Carrying Out the Invention

While any particulate based material compatible with the phagocytic cell system may be used, organically-derived polymeric material and particularly polyacrylamides (for example, Bio-Rad® particles, Richmond, California) and particulate zymosan have been found to be particularly suitable.

In order for this system to function properly, it is necessary to coat protein onto the

particulate material. This coating may be accomplished by actual chemical linkage of the protein to the particle or by simple electrostatic adsorption. Any suitable protein or mixture of proteins may be used to thus sensitize the beads. Suitable proteins include purified human immunoglobulin G or human or animal serum.

Next, the luminescent chemical is either chemically or by adsorption, coated on or admixed with the protein coated particles. While any luminescent chemical that reacts with oxygen intermediates directly or indirectly generating light can be used, luminol (e.g. available from Eastman Organics, Rochester, New York) has been found to be particularly suitable. If the polymer bead is used, the luminol is dissolved in an aqueous buffered solution, and preferably reacted to form a reactive azo-intermediate. The azo-intermediate is then reacted with the protein coated polymeric particles. This results in an azoluminol-protein adduct as well as electrostatically bound luminol-polyacrylamide. The resulting particles are washed, resuspended to a final concentration, filled into vials and lyophilized. The material derived from this process yields a stable, homogeneous product.

The luminol can also simply be admixed with a suspension of zymosan particles previously coated with a suitable protein. The resulting admixture is filled into vials and lyophilized. The material derived from this process yields a stable, homogeneous product.

The lyophilized product described above is, after reconstitution with water, ready to use in the assay method.

For the assay method using polymeric particles, 1,000,000 to about 2,000,000 polyacrylamide particles in 50 microliters of buffer are mixed with about 200,000 purified phagocytic cells in 100 microliters of buffer. Two exemplary assay methods used in conjunction with the zymosan particles are: (1) whole blood is diluted 1:3 (one part anticoagulated blood plus two parts buffer). Fifty microliters of this dilution are mixed with 200 microliters of the coated zymosan containing luminol. Each milliliter of zymosan mixture contains approximately 50,000,000 particles; and (2) Fifty microliters of zymosan suspension is mixed with 100 microliters buffer containing approximately 200,000 purified phagocytic cells. The light generated from these mixtures is monitored periodically over time and measures the phagocytic and biochemical activity of the phagocytic cell preparation. Cells which may be evaluated by this technique include neutrophils, monocytes and alveolar macrophages. The first two of these cell types are obtained from whole blood placed on a density gradient such as Ficoll™-Hypaque™ and centrifuged. These cells band in the gradient and are thus purified. Macrophages are obtained from lung washings and do not require purification. In the following examples, neutrophils are used.

Example 1

200 mg of the above-described polyacrylamide beads having a 2–9 micron diameter are suspended in 20 ml of water. To this suspension, 10 mg of human immunoglobulin G (IgG) are added. The protein bead suspension is gently mixed and then chilled to 2°C–8°C. 40 mg of 1-ethyl-3,3 dimethyl aminopropyl carbodiimide hydrochloride is added. Six hours later, 150 mg of glycine is added. The mixture is stirred overnight at 2°C–8°C. The next day, the suspension is washed by centrifugation with phosphate buffered saline, 1 M sodium chloride in phosphate buffered saline and finally 0.005 M phosphate buffer. The beads are centrifuged again and resuspended in 8.5 ml of 0.5 M pH 8.5 borate buffer.

A diazonium salt of luminol is prepared by suspending 200 mg luminol in 20 ml 2.4 N HCl. The mixture is chilled on ice. 2.0 ml 100 mg per ml sodium nitrite is added, mixed, followed rapidly by the addition of chilled buffer. 50 ml 0.5 m pH 8.5. The pH is observed and adjusted with 10 N sodium hydroxide to pH 7.1 ± 0.1. 1.0 ml of the diazonium salt of luminol is then added to the 8.5 ml of resuspended beads described above and the pH adjusted to pH 8.5. The suspension is mixed for three hours in the dark at 2°C–8°C.

The diazotized bead suspension is then dialyzed against several changes of 0.2 M pH 8.0 borate buffer at 2°C–8°C. Dialysis proceeds for at least 24 hours.

The beads are then washed by centrifugation using 0.2 M pH 8.0 borate buffer until the supernatant has less than 1.0% of the initial light output of the total suspension, i.e. greater than 99% of luminol associated with the beads.

The beads are then centrifuged once again. The beads are resuspended in phosphate buffered saline. The bead concentration is adjusted to approximately 50,000,000 beads per milliliter as determined with a hemocytometer. The suspension is then filled into vials in 1.0 ml portions and lyophilized to less than 5.0% residual moisture as determined by Karl Fisher titration.

To use in the phagocytic assay, a vial is reconstituted by the addition of 1.0 ml purified water.

Example 2

Zymosan A (Sigma Chemical Co., St. Louis, Missouri) is suspended in phosphate buffered saline to a concentration of 24 mg per ml. To this suspension is added an equal volume of 60% normal rabbit serum diluted in phos-

phate buffered saline. The suspension is incubated for one hour at 37°C in a shaking water bath. The suspension is then centrifuged, the supernatant discarded and the thus treated Zymosan A (pellet) resuspended in a small volume of phosphate buffered saline. The suspension is then drawn through a syringe needle (18–26 gauge) to homogenize the suspension. Finally sufficient phosphate buffered saline is added to four times the original volume of suspension resulting in an approximate 6 mg per ml suspension. This suspension is then passed through a glass wool plug to entrap any remaining large clumps of zymosan.

A stock solution of luminol is prepared by dissolving it in 0.01 N sodium hydroxide. An aliquot of this stock solution is then added to a solution containing 20% fetal calf serum in phosphate buffered saline resulting in a luminol concentration of 14.4 micrograms per ml.

To one volume of zymosan suspension is added one volume of luminol fetal calf serum solution. The resulting suspension is mixed, filled into vials in 2.0 ml portions and lyophilized to less than 5% residual moisture as determined by Karl Fisher titration.

To use in the phagocytosis assay, a vial is reconstituted with 2.0 ml purified water.

Example 3

Several children previously diagnosed as having chronic granulomatous disease (CGD) were examined using the reagents and assay method described above. This disease was selected since the dysfunction is understood. CGD is caused by the absence of certain enzymes found in phagocytic cells of the blood. Although the phagocytic cells are capable of engulfing bacteria (particles), the cells are unable to inactivate or kill the bacteria since the phagocytic cells lack the ability to generate high energy oxygen intermediates. As such, they cannot cause the oxidation of luminol so engulfed by the cells, hence no detectable light response is observed. Clinically, these children typically present or manifest this decrease as a severe reduction in their ability to resist infection. See Figs. 1–3 for typical response. When these children were tested along with apparently healthy control subjects, they produced no light from luminol oxidation, although they engulfed particles as effectively as the controls.

Example 4

A group of children previously diagnosed as having asthma were examined as related to their phagocytic/biochemical response of phagocytic cells. Approximately half the children were receiving therapeutic doses of theophylline; the remaining children had not been placed on this medication. Theophylline is thought to have an effect on cyclic adenosine monophosphate (c-AMP) which is known to exert regulatory control over certain biochemical actions of phagocyticells. See Figs. 4–6. The children receiving theophylline generally demonstrated reduced levels of oxygen intermediate production as quantitated by reduced luminol oxidation and light production.

Such methods as described have particular utility for the quantification of infection resistance in terms of phagocyte activity. However, such process also has application in medical diagnosis, environmental immunotoxicology, pharmacology, such as for monitoring toxicity of chemotherapy and radiation therapy patients, as examples. It can be used for the evaluation of immunocompetency, certain blood serum protein defects, etc. As a research tool, it can be used to test pharmaceutical compounds and their effect on phagocytes, effects of pollutants on phagocytic cells and the effect of toxic compounds on animals and humans.

Although this invention has been described with respect to detailed embodiments thereof, it will be understood by those skilled in the art that various changes in form and detail thereof may be made without departing from the spirit and scope of the claimed invention.

CLAIMS

1. A method of measuring the ability of an organism to resist infection comprising taking a sample of blood from the organism, separating the phagocytic cells from the blood, adding to the phagocytic cells particles coated with protein having a luminescent chemical bonded thereto, causing the phagocytic cells to engulf the particles thus activating the cells' biochemical mechanism which reacts with the luminescent chemical generating light which is measured on a luminometer.

2. The method of claim 1 wherein the particles are polymeric beads having diameters of about 2 microns to about 9 microns.

3. The method of claim 2 wherein the beads are polyacrylamide.

4. The method of claim 1 wherein the particles are zymosan particles.

5. The method of claim 1 wherein the luminescent chemical is luminol.

6. A phagocytic composition comprising polyacrylamide particles having a diameter of about 2 microns to about 9 microns coated with a layer of serum protein having a layer of luminol bonded thereto.

7. The composition of claim 6 in lyophilized form.

8. A phagocytic composition comprising zymosan particles coated with a layer of serum protein and luminol.

9. The composition of claim 8 in lyophilized form.

10. A method of making a phagocytic reagent comprising coating particulate material about 2 microns to about 9 microns in

diameter with a layer of protein and luminescent chemical, and lyophilizing the coated particles to a stable, homogeneous product.

11. The method of claim 10 wherein the protein layer is applied first and luminescent chemical applied thereto.

12. The method of claim 10 wherein the luminescent chemical is admixed with the protein prior to application to the particulate material.

13. The method of claim 10 wherein the luminescent chemical is luminol.

14. The method of claim 10 wherein the particulate material is polyacrylamide or zymosan.

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